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## Hybrid thermochemical processing: fermentation of pyrolysis-derived bio-oil

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## **Abstract**

Thermochemical processing of biomass by fast pyrolysis provides a non-enzymatic route for depolymerization of biomass into sugars that can be used for the biological production of fuels and chemicals. Fermentative utilization of this bio-oil faces two formidable challenges. First is the fact that most bio-oil-associated sugars are present in the anhydrous form. Metabolic engineering has enabled utilization of the main anhydrosugar, levoglucosan, in workhorse biocatalysts. The second challenge is the fact that bio-oil is rich in microbial inhibitors. Collection of bio-oil in distinct fractions, detoxification of bio-oil prior to fermentation and increased robustness of the biocatalyst have all proven effective methods for addressing this inhibition.

**Keywords** levoglucosan; bio-oil; ethanol; lipids; inhibition; toxicity; furfural; acetic acid

## Introduction to thermochemical processing

As a temporary storage unit of sunlight-derived energy and atmospheric carbon, biomass is an excellent source of carbon and energy for the production of biorenewable fuels and chemicals. However, the cost- and energy-efficient release of fermentable sugars from this biomass is challenging, largely due to the complex structure of lignocellulose. Existing techniques for biomass deconstruction can be generally categorized as either biochemical or thermochemical, with chemical and physical processing also playing a significant role. While biochemical processing generally uses enzymes to deconstruct biomass and microorganisms to synthesize products, thermochemical processing employs heat to deconstruct biomass and chemical catalysis for product formation. A third processing method, referred to here as hybrid processing, employs both thermochemical and biochemical steps (Figure 1). Thermochemical processing of lignocellulosic biomass by pyrolysis readily overcomes its recalcitrance while biological utilization of the pyrolysate provides high product selectivity.

Pyrolysis is the thermal decomposition of biomass in the absence of oxygen to produce an energy-rich liquid (bio-oil), a flammable gas (syngas), and a carbon-rich solid (biochar). When optimized for the production of bio-oil, the process is known as fast pyrolysis. Some advantages of fast pyrolysis relative to biochemical processing of biomass include the rate of processing (requires seconds rather than hours or even days), the ability to utilize both the carbohydrate and lignin, and flexibility in the composition of biomass that can be processed (Venderbosch 2011).

A recent study compared the fast pyrolysis route to biofuels with enzymatic hydrolysis and gasification routes (Anex 2010), assuming the processing of \$75 per ton herbaceous feedstock at a scale of 2000 tons per day. Near-term cost was \$2-3 per gallon gasoline equivalent (gge) for pyrolysis-derived fuel, \$4-5 per gge for gasification-based fuel, and \$5-6 per gge for cellulosic ethanol via enzymatic hydrolysis. A separate economic assessment found the cost of ethanol from fermentative utilization of bio-oil to be comparable to the cost from either acid- or enzyme-mediated hydrolysis of woody biomass (So 1999).

While this article focuses on biological utilization of bio-oil, we will briefly describe the other products of thermochemical biomass degradation, syngas and biochar. Syngas mainly contains hydrogen and carbon monoxide, with lesser amounts of methane, carbon dioxide and water. While it can be burned for heating or power generation, its heating value ( $\sim 6 \text{ MJ kg}^{-1}$ ), is lower than that of natural gas ( $\sim 54 \text{ MJ kg}^{-1}$ ) (Laird et al. 2009). Syngas can also be used as feedstock for either chemical or biocatalytic production of fuels and chemicals. In the biocatalytic route, microorganisms use syngas as substrate for

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production of compounds such as methane, acetate, ethanol, butanol, and biopolymers (Munasinghe and Khanal 2010).

Biochar can be used as a fuel or for carbon sequestration and soil improvement. It can be burned as a replacement for pulverized coal but, because of its resistance to chemical and microbial breakdown, can also serve as a long-term means of carbon sequestration. Application of this biochar, along with compost or fertilizers, to soils can increase crop productivity (Blackwell et al. 2009).

As with syngas and bio-char, bio-oil has multiple applications. Extensive catalytic applications have been described elsewhere. Here, we discuss progress in the fermentative utilization of bio-oil. To the best of our knowledge, this is the first review article published on this topic.

### **Bio-oil-associated sugars: levoglucosan**

Fermentation feedstocks need to be rich in substrates for the biocatalyst. Historically, the sugar content of bio-oil was thought to be low. However, it has been shown that many of the previously-identified water-soluble components of bio-oil are actually sugars (Patwardhan et al. 2009; Patwardhan et al. 2010). Biomass pretreatment can further enhance the yield of sugars. Under ideal conditions, cellulose depolymerizes at high yields to 1,6-anhydro- $\beta$ -D-glucose, an anhydrosugar known as levoglucosan. However, the low yield of levoglucosan in bio-oil produced from untreated biomass is due to the presence of alkali or alkaline cations which could retard the formation of levoglucosan from cellulose via interaction with the terminal chain end of cellulose (Patwardhan et al. 2009; Patwardhan et al. 2010). Studies on woody biomass demonstrated that cation removal increased levoglucosan content approximately 10-fold in bio-oil, from 3.0 to 30.4 wt% on a moisture-free basis (Scott et al. 1989). Other efforts, such as collection of the pyrolysate in fractions, can increase the substrate availability (Westerhof et al. 2011; Pollard et al. 2011). Thus, appropriate processing can result in bio-oil that is rich in levoglucosan and other sugars that could be fermented to biorenewable fuels and chemicals. Unfortunately, most workhorse biocatalysts lack the ability to directly metabolize levoglucosan.

Levoglucosan can be converted to glucose by acid hydrolysis or catalysis, but these additional processing steps represent additional cost. Instead, it is desirable to work with organisms that can directly metabolize levoglucosan. While it was originally thought to be a scarce substance in nature, levoglucosan has been found in abundant quantities where forest fires or other types of biomass burning incidents have occurred (Prosen et al. 1993). Searches for levoglucosan utilizers have identified several microorganisms that can use levoglucosan as sole carbon and energy source. For example, *Aspergillus terreus* K26 metabolizes levoglucosan to produce itaconic acid (Nakagawa et al. 1984). Similarly, *Aspergillus niger* This is a manuscript of an article from *Applied Microbiology and Biotechnology* 91 (2011): 1519, doi: 10.1007/s00253-011-3495-9. Posted with permission. The final publication is available at Springer via <http://dx.doi.org/10.1007/s00253-011-3495-9>.

CBX 209 produces citric acid from levoglucosan (Zhuang and Zhang 2002). In both of these examples, the yield and the rate of fermentative production from levoglucosan are comparable to glucose. These findings suggest that levoglucosan can be fermented as effectively as conventional hexose sugars.

Biochemical analysis of the levoglucosan utilization pathways revealed that in yeast, levoglucosan is introduced into the general glycolytic pathway by Mg-ATP-dependent levoglucosan kinase (LGK), producing glucose-6-phosphate (Kitamura and Yasui 1991; Kitamura et al. 1991). Contrastingly, some soil-derived bacteria use a pathway that begins with levoglucosan dehydration (Kitamura et al. 1991).

The *A. niger* CBX-209 LGK was subjected to extensive biochemical characterization (Zhuang and Zhang 2002). This analysis identified the optimal temperature of 30°C and an optimal pH of 9.3, though the enzyme is stable at pH 6 – 10. This same analysis showed a strict substrate preference for levoglucosan. The enzyme is inhibited by Mg-ADP, HgCl<sub>2</sub> and CoCl<sub>2</sub>, but not by glucose-6-phosphate. One potential problem is the relatively high K<sub>m</sub> of LGK for levoglucosan: 71.2mM in *A. niger* (Zhuang and Zhang 2002) and 68 mM in *L. starkeyi* (Dai et al. 2009). This high K<sub>m</sub> appears to result in incomplete substrate utilization (Dai et al. 2009; Layton et al. 2011), decreasing the overall product yield.

Given the status of *E. coli* as a premier industrial workhorse and producer of biorenewable chemicals, it is desirable to engineer this organism for levoglucosan utilization. The fungal LGK was cloned into *E. coli* from an *A. niger* genomic library, but the resulting enzyme activity was low (Zhuang and Zhang 2002). Isolation of LGK from *L. starkeyi* YZ-215 and its expression in *E. coli* enabled utilization of levoglucosan as sole carbon source (Dai et al. 2009). Our own efforts have demonstrated that existing commercially successful biocatalysts can be modified for levoglucosan utilization (Layton et al. 2011).

These works demonstrate that levoglucosan, an abundant component of bio-oil, can be used by biocatalysts as a carbon and energy source (Table 2) and that the levoglucosan utilization pathway can be functionally expressed in standard biocatalysts.

### **Addressing Contaminant Toxicity**

While bio-oil is a tantalizing substrate for the production of biorenewable fuels and chemicals, even early studies noted the inhibitory effect that it has on biocatalysts. Nearly twenty years ago, it was noted that several fungal species could grow in bio-oil that had been treated with activated charcoal but not in the raw aqueous bio-oil extract (Prosen et al. 1993).

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This inhibitory effect can be attributed to the undesirable “contaminants”, such as furans, phenols and organic acids. Table 1 lists contaminant compounds for which the inhibitory concentration is known for *E. coli*. Some of these compounds, such as furfural and acetic acid, have been extensively studied as biocatalyst inhibitors and are discussed briefly below. However, bio-oil also contains many other compounds for which the mechanism of inhibition has not been characterized. Thus, the utilization of bio-oil as a fermentation substrate depends on more than just the production of bio-oil with high sugar content; the inhibitory properties of these contaminant compounds must also be addressed.

As demonstrated by the activated charcoal treatment, one approach to mitigating this inhibition is to add a detoxification step prior to fermentation. Major detoxification processes reported for bio-oil include solvent extraction, adsorption on activated carbon, and over-liming (Lian et al. 2010; Chan and Duff 2010) (Table 2). Another approach of the pre-treatment strategy is to collect the bio-oil in distinct fractions and use the fraction(s) rich in substrates but depleted in inhibitory contaminants (Westerhof et al. 2011; Pollard et al. 2011) (Figure 2).

A parallel approach to the reduction of toxicity of the bio-oil is to use a biocatalyst that is tolerant of these inhibitory contaminant compounds. Tolerance is a complex phenotype and tolerance to complex, highly-variable bio-oil is even more challenging. If the inhibition could be attributed to a single inhibitory compound and the mechanism of inhibition by that compound were known, rational steps could be implemented to improve tolerance. However, given that many inhibitory compounds are present in bio-oil at or above their inhibitory concentration, it is difficult to attribute bio-oil toxicity to a single compound. Additionally, many of these inhibitory compounds are known to act synergistically (Zaldivar and Ingram 1999; Zaldivar et al. 1999; Couallier et al. 2006), further complicating identification of the major mechanism of inhibition.

There are many randomized approaches for improving biocatalyst tolerance to inhibitory compounds when the mechanism of inhibition is not known. Metabolic evolution has been utilized in the development of biocatalysts capable of fermenting bio-oil (Table 2). Specifically, yeast was adapted to bio-oil that had been detoxified by solvent extraction and then hydrolyzed to convert anhydrosugars to glucose. The ethanol yield of the adapted strains was increased 39% relative to the non-adapted strain (Chan and Duff 2010). However, there was no data presented about the mutations that conferred bio-oil tolerance and therefore the results are difficult to extrapolate to other yeast strains or to other organisms.

## **Model Contaminants**

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As mentioned above, some of the compounds present in bio-oil have been extensively studied, motivated by the fact that these compounds are also present in biomass hydrolysate. Acetic acid content can be greatly decreased by collecting bio-oil in distinct fractions (Westerhof et al. 2011; Pollard et al. 2011), but the aldehyde and phenolic compounds are still problematic. The results of previous aldehyde studies can possibly provide insight into bio-oil toxicity, and a few studies are highlighted here.

Furfural toxicity in *E. coli* was attributed to NADPH depletion by the furfural reductase enzyme YqhD, which converts furfural to furfuryl alcohol (Miller et al. 2009a; Miller et al. 2009b). Silencing of this enzyme increased not only tolerance to furfural but also to the closely-related 5-hydroxymethyl furfural by increasing NADPH availability for biosynthesis (Miller et al. 2010). Similar effects were observed in yeast, where furfural and 5-hydroxymethyl furfural were both consumed during the period of growth inhibition (Taherzadeh et al. 1999, 2000). Thus, one possible method of dealing with the toxic aldehydes present in bio-oil is to mitigate NADPH depletion by either silencing the respective aldehyde reductase or by a general increase in NADPH availability.

While current research efforts can utilize existing toxicity data, results from Lian *et al* suggest that some of the most toxic bio-oil components are those with little available toxicity data, such as eugenol, acetol and vanillin (Lian et al. 2010). Thus, additional studies of these compounds could be beneficial for improving bio-oil utilization.

## **Concluding Remarks**

In order for bio-oil fermentation to move forward, additional progress needs to be made both in detoxification of the bio-oil and in development of biocatalysts that are robust to the inhibitory contaminant compounds. Metabolic evolution is a useful tool for increasing robustness, but the resulting tolerant strains need to be reverse engineered, so that insights about the mechanisms of tolerance can be applied to other biocatalysts.

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